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This current proposal is based on our previous observations that: (1) Interferons (IFNs) are capable of exerting growth inhibition and anti-tumor effects on human cancer cells; and (2) p202 expression alone is sufficient to suppress both cell growth and tumor development of human prostate and breast cancer cells. To further investigate the anti-tumor activity of p202 on prostate cancer and to develop a p202 gene therapy for prostate cancer, we have proposed three specific aims to accomplish our objectives.
Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells;
Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer;
Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclinical gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer animal model.
Success of those aims will constitute a scientific basis for p202-associated anti-tumor effect on prostate cells and will enable us to develop a novel p202 gene therapy strategy against prostate cancer.

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INTRODUCTION:

The interferon family (IFN) is composed of three classes:α, β and γ (1). The IFN family not only plays an integral role in host defense system against certain tumors and foreign antigens such as viruses, bacteria and parasites; but also possesses immunomodulatory and cell growth-inhibitory activities. However, the molecular mechanisms involved in IFN's anti-tumor activity are remained elusive. In a recent study, several IFN-inducible proteins are implicated in the process of tumor suppression (2). Moreover, based on DNA analysis, 19 out of 95 IFN-inducible genes are differentially downregulated during prostate tumor progression (3). The anti-cellular function of IFNs has been attributed to their abilities to induce G_1 phase arrest in cell cycle (4-6). P202, an IFN-inducible gene is a primarily nuclear 52kd phosphoprotein, has been shown to have a growth retardation function that was presumably accomplished by its ability to bind several cell-cycle regulatory proteins such as E2Fs, AP1, NFκB and pRb, resulting in the failure of S phase entry (7-9). Using p202 as a therapeutic agent, we have demonstrated that the multiple anti-tumor activities in human cancer xenograft models including breast and pancreatic cancers (10-11). Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibits cellular proliferation and suppresses transformation phenotype *in vitro* (12). Our ultimate goal is to develop a gene therapy strategy that would specifically deliver p202 to the prostate cancer cells so that the "normal cells" would not be affected by such treatment. To accomplish our goal, three specific aims are proposed (see below). The

success of those aims will constitute a scientific basis for p202-associated anti-tumor effect on prostate cancer cells and will enable us to develop a novel p202 gene therapy strategy against prostate cancer.

BODY:

A. SPECIFIC AIMS: (NO CHANGES)

Specific Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells.

Specific Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer.

Specific Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

B. STUDIES AND RESULTS:

Specific Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells.

To investigate the anti-tumor ability of p202, we have established two p202 stable transfectants, p202-1 and p202-1 in human prostate cell line, PC3. Two transfectants were injected into nude mice separately and produced smaller or no tumors after sixteen-

weeks post-implantation (Appendix Figure 1A). Furthermore, we performed *ex vivo* tumorigenicity assay in which the PC-3 cells were transiently transfected with CMV-p202 using two different liposome delivery systems PEI or LPD-1. Then, the p202 transfected PC-3 cells were employed to generate subcutaneous xenografts in nude mice. The p202 transfected PC-3 showed no detectable tumors after ten days, regardless of the liposome delivery systems (Appendix Figure 1B). Our studies provide a scientific basis for developing a p202-based gene therapy in orthotopic human prostate cancer xenograft model. Further investigation will be addressed in Specific Aim 3.

Specific Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer.

To investigate the molecular mechanisms of the p202-mediated growth inhibition and tumor suppression in prostate cancer cells, we examined: 1) pRb phosphorylation was involved in p202-mediated growth arrest since IFN treatment increases the active (hypophosphorylated) form of pRb (18-20); and 2) other cell cycle regulatory genes, which were identified by DNA array analysis, may be responsible for the p202-mediated growth retardation and tumor suppression. We have analyzed phosphorylation status of pRb in both PC3 and p202-expressing PC3 cells. The p202-expressing cells has elevated level of active (hypophosphorylated) form of pRb as compared to parental cells (Figure 4A). It may suggest that the active form of pRb inhibits E2F transactivation function, which results in inhibiting E2F-mediated transcription of S phase genes, and therefore arresting in G_1 of cell cycle. To further investigate whether other cell cycle regulatory genes may be responsible for p202-mediated anti-growth ability, we examined DNA

array data from PC3 and p202-expressing PC3 cells. We have identified two potential candidates, cyclin B and p55cdc, which expression are downregulated in p202-expressing PC3 cells. It should be worth noting that cyclin B is known to involve in G2/M phase transition (21), and p55cdc is required for normal metaphase-to-anaphase transition in the late mitotic events (22). It is possible that the p202-associated downregulation of cyclin B and p55cdc may contribute, in part, to the p202-mediated growth arrest.

Specific Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

To achieve prostate-specific p202-mediated anti-tumor activity, we first examined an AR (Androgen Receptor)-responsive promoter, ARR_2PB which contains two copies of androgen responsive regions (ARR) located upstream from a minimum PB (probasin), therefore, it is highly responsive to androgen-dependent transcriptional activation (13-15). We have constructed ARR_2PB-Luc (luciferase reporter gene) and transfected into two prostate cell lines LNCaP (with endogenous AR expression) and PC3 (without endogenous AR expression). We have demonstrated that the relatively luciferase activity is the highest in the AR-positive LNCaP cells, but not in AR-negative PC-3 cells (Appendix Figure 2A). These results suggest that ARR_2PB promoter activity is indeed AR-dependent, and is feasible to direct AR-specific gene expression in prostate cancer cells. To further investigate whether the ARR_2PB promoter could direct prostate-specific p202 expression and tumor suppression *invivo*, we have generated a p202 expression vector driven by ARR_2PB promoter (ARR_2PB-p202), and established the orthotopic

prostate cancer xenograft model, i.e. mice-bearing LNCaP cell tumors orthotopically were treated intravenously with ARR_2PB-p202 or ARR_2PB-Luc. The tumor-bearing mice treated with ARR_2PB-p202 has a prolonged survival rate compare to ARR_2PB (Appendix Table 1). In addition, the ARR_2PB-p202-treated tumors were significantly reduced in size than those treated by the control vector, ARR_2PB-Luc (Appendix Figure 2B). Interestingly, the ARR_2PB-p202 expression was readily detected in tumors (Appendix Figure 3A), however, abundant intracytoplamsic expression of p202 which primarily is a nuclear protein, was observed (Appendix Figure 3A left panel). The reason for cytoplasmic localization of p202 in the tumors is not clear. It is likely that the highly exogenous expression of ARR_2PB-p202 may cause accumulation of p202 in the cytoplasm. Alternatively, since the IFN-induced p202 was in the cytoplasm for 30-36 hours before translocated into nucleus (16). It is possible that p202 could still remain in the cytoplasm 20 hours post ARR_2PB-p202 treatment. To examine whether the ARR_2PB-p202 expression is prostate-specific, we have analyzed various organs with immunohistochemical staining. There was no extraprostatic expression of p202 except the reticuloendothelial cells of lung and liver (Appendix Figure 3B). The p202 expressed in reticuloendothelial cells of lung and liver may be due to endogenous expression of p202, since all 200 amino-acid protein family members are expressed in hematopoietic cells (17). In summary, our studies strongly suggest that systemic treatment of ARR_2PB-p202 could result in prostate- and AR-specific anti-tumor activity in prostate cancer. We will continue to assess the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

KEY RESEARCH ACCOMPLISHMENTS:

- P202 reduces the tumorigenicity of PC-3 cells *ex vivo*.
- P202 enhances hypophosphorylated pRb to impede S phase entry of cell cycle in human prostate cancer cells, PC-3.
- P202 also reduces expression of G_2/M cell cycle regulators, cyclin B and p55cdc in human prostate cancer cells, PC-3.
- ARR_2PB promoter activity is AR-dependent in human prostate cancer cells with endogenous AR (Androgen Receptor) expression, LNCaP.
- ARR_2PB promoter directs prostate-specific expression of p202 gene *in vivo*.
- Systemic treatment with ARR_2PB-p202 suppresses tumor growth *in vivo*.

REPORTABLE OUTCOMES:

We are in the process to submit a manuscript entitled "Prostate Specific Anti-tumor Activity by Probasin-Directed p202 Expression". A draft of manuscript is attached.

CONCLUSIONS:

In this report, we have demonstrated that either CMV-p202/PEI or CMV-p202/LPD-1 expression reduces the tumorigenicity of prostate cancer cells *ex vivo*. In addition, we have constructed a prostate-specific p202 expression via ARR_2PB promoter which is

androgen receptor-dependent. The expression of p202 driven by a composite probasin promoter, ARR_2PB is prostate-specific and suppresses tumor growth via systemic treatment *in vivo*. Our results suggest that the feasibility of using a tissue-specific promoter to achieve p202-mediated anti-tumor activity in prostate cancer. Furthermore, to investigate molecular mechanisms of p202's anti-tumor activity, we have identified that p202 may associate with upregulating hypophosphorylated pRb and downregulating the expression of cyclin B and p55cdc. Thus, our study suggests that the p202's anti-tumor activity may involve in deregulating of the G_2/M phase of cell cycle (i.e. by down-regulating expression of G_2/M cell cycle regulators, such as cyclin B and p55cdc), in addition to G_1 arrest (i.e. by targeting E2F/pRb pathway in G_0/G_1 transition.)

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APPENDIX:

Prostate Specific Anti-tumor Activity by Probasin Promoter-Directed p202 Expression

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Running Title: Prostate specific anti-tumor activity by p202

Key words: p202, tumorigenecity, probasin, cyclin B, p55cdc

CONDENSED ABSTRACT

Systemic administration of a p202 expression vector driven by a prostate-specific probasin (ARR_2PB) gene promoter (ARR_2PB-p202) resulted in prostate-specific tumor suppression in prostate cancer xenograft model. We also showed that p202 expression was accompanied by down-regulation of G2/M phase cell cycle regulators, cyclin B and p55cdc, suggesting G2/M deregulation may also contribute to the p202-mediated inhibition of prostate cancer cell growth.

ABSTRACT

BACKGROUND. p202, an interferon inducible protein, arrests cell cycle at G1 phase and thus retards cell growth. We previously showed that ectopic expression of p202 in human prostate cancer cells rendered growth inhibition and suppression of transformation phenotype *in vitro*.

METHODS. p202 stable prostate cancer cell lines were used in tumorigenicity assay to examine their *in vivo* transformation potential. In addition, *ex vivo* treated prostate cancer cells with p202 expression vector were tested for their tumorigenicity. A prostate-specific probasin (ARR_2PB) gene promoter was used to direct p202 expression (ARR_2PB-p202) in androgen receptor (AR)-positive manner. ARR_2PB-p202/liposome complex was systemically administered into mice bearing AR-positive prostate tumors to monitor the anti-tumor activity. DNA array and western blot analysis were used to identify the genes regulated by p202 expression.

RESULTS. Prostate cancer cells with stable expression of p202 are less tumorigenic *in vivo* and *ex vivo* treatment with p202 expression vector suppress the growth of prostate

tumors. Parenteral administration of an ARR_2PB-p202/liposome preparation led to prostate-specific p202 expression and tumor suppression in prostate cancer xenograft model. The expression of p202 was accompanied by down-regulation of G2/M phase cell cycle regulators, cyclin B and p55cdc.

CONCLUSIONS. The current study suggests that p202 suppresses prostate tumor growth, and that a prostate-specific anti-tumor effect can be achieved by systemic administration of liposome-mediated delivery of ARR_2PB promoter-driven p202 expression vector. In addition to G1 phase arrest, G2/M deregulation may also contribute to the p202-mediated inhibition of prostate cancer cell growth.

INTRODUCTION

The IFN family of cytokines plays a crucial role in host defense system against viral, bacterial and parasitic infections and certain tumors. In addition, they also possess immunomodulatory and cell growth-inhibitory activities. There are three classes of interferon: a, b, and g [1]. The mechanism involved in tumor suppressor activity of IFNs has not been well established. However, several IFN-inducible proteins were implicated in the process of tumor suppression [2]. Consistent to that notion, a recent report based on DNA array analysis indicates that 19 out of 95 differentially down-regulated genes associated with prostate tumor progression are, in fact, IFN-inducible genes [3]. The anti-cellular function of IFNs has been attributed to their abilities to induce G1 phase arrest in cell cycle [4-6]. Human prostate cancer cells are also sensitive to the anti-mitotic action of

IFNs [7, 8]. Recent studies demonstrated inhibitory effect of IFN-a on growth [9-11] and colony formation [12] in several human prostate carcinoma cell lines.

Besides the therapeutic effects of IFNs in certain clinical settings, there were also undesirable side effects viz. fever, chills, anorexia, and anemia associated with high dose IFN which is often required to obtain therapeutic response [13, 14]. This has impeded IFN as an effective anti-cancer agent. In an attempt to circumvent this disadvantage and to harvest the benefit of IFN treatment, we explored the possibility of using an IFN-inducible protein, p202 [15], as a potential therapeutic agent. p202 belongs to murine 200 amino-acid protein family. Although the physiological function of p202 is not well defined, the experimental evidence gathered so far suggests its role in cell cycle control, differentiation, and apoptosis [16, 17]. In particular, ectopic p202 expression in cells resulted in retardation of growth that is thought to be mediated by E2F/Rb pathway leading to G1 arrest [18, 19].

Using p202 as a therapeutic agent, we have demonstrated the multiple anti-tumor activities in human cancer xenograft models including breast and pancreatic cancers [20, 21]. Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibited cellular proliferation and suppressed transformation phenotype *in vitro* [22]. However, it has not yet been determined whether p202 expression inhibits the tumorigenicity of prostate cancer cells and whether the p202-based gene therapy is feasible in human prostate cancer

xenograft model. In this report, we show that p202 expression reduces the tumorigenicity of prostate cancer cells. Using a p202 expression vector driven by ARR_2PB promoter [23-25], we show prostate-specific expression of p202 and tumor suppression. Interestingly, we show that the anti-cellular effect involves deregulation of G2/M phase of cell cycle.

MATERIALS AND METHODS

Cell lines and Plasmids.

LNCaP, MCF-7, PC-3, and four p202-expressing PC-3 clones, i.e. p202-1, -2, -3, and -4 [22] were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum. The p202 expression vector, CMV-p202 [19], is driven by CMV promoter. To construct the ARR_2PB-luc vector, the ARR_2PB promoter element (468 bp), in pBlueScript II SK+ vector [23], was ligated into the KpnI/Sac I site of the PGL3-enhancer vector (Promega, Madison, WI). The ARR_2PB-p202 was generated by replacing the luciferase gene in the ARR_2PB-luc with the p202 coding sequence obtained from CMV-p202 vector [19] by Bam HI digest. The correct orientation was confirmed by unique restriction enzyme digestion.

Subcutaneous and Ex Vivo Tumorigenicity Assays. PC-3 vector control (pcDNA3-pool), p202-1, and p202-2 cells (1 x 10^6 each) in 200 µl of PBS were injected subcutaneously in 4-5-week old nude mice on both sides of the abdomen. Tumor sizes were measured with a caliper once a week and tumor volume was calculated using the formula: Vol. = S×S×L/2, where S = the short length of the tumor in cm, and L = the long length of the tumor in cm. For ex vivo experiment: PC3 cells growing in 100 mm dishes were transfected with 10 μg of CMV-p202 DNA complexed with either 22.5 μg PEI

(polyethylenimine) for 45 min. or with the cationic liposome LPD-1 (8.8 μg lipid, 8 μg protamine sulfate) for 4.5 hr. PC3 cells were mock transfected with either CMV-p202 alone or PEI or LPD-1 alone. After transfection, the cells were washed and incubated for an additional 18 h in complete media. Cells were then trypsinized, washed in PBS, counted, and $1x10^6$ cells were inoculated s.c. in two sites on the flanks of male nude mice. Tumor size was measured weekly and volume calculated.

Transfection and Luciferase Assays. Human prostate cancer cell lines LNCaP and PC3, and a human pancreatic cancer cell line (Panc-1) were used for the reporter assay. 2×10^6 cells were plated into 6-well plated the day before transfection. 0.5 μg of ARR2-PB-luc plasmid, 0.5 μg of CMV-luc plasmid and 0.05 μg of pRL-TK were transfected into cells using SN2 liposome. Cells were harvested 36 h after transfection. The luciferase activity was determined using the dual luciferase protocol (Promega) with a luminometer.

Immunohistochemistry. The avidin-biotin peroxidase complex technique used in this study is modified from that described previously [26]. Briefly, formalin fixed tissue sections were deparaffinized and dehydrated in ascending grades of ethanol. The sections were treated with 0.05% trypsin for 15 min, blocked in 0.3% hydrogen peroxidase in methanol for 15 min followed by treatment with 1% (v/v) normal horse serum for 30 min. The slides were incubated overnight at 4°C with anti-p202 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, California) at 1:25 dilution. After liberal washing with phosphate buffer saline (PBS), the slides were

incubated with biotinylated rabbit anti-goat Ig G at 1:200 dilution in PBS for 60 min at room temperature. The slides were subsequently incubated for 45 min. at room temperature with the avidin-biotin-peroxidase complex diluted 1:100 in PBS. The product of enzymatic reaction was visualized with 0.125% aminoethylcarbazole, which gives a red colored reaction product. For counter staining Mayer's hematoxylin was used.

Systemic Gene Therapy in Human Prostate Cancer Xenograft Model. Athymic nude mice (nu/nu) opened through a single mid-ventral incision under sedation and prostate gland exposed. An aliquot of 30 μl PBS containing 2 x 10^6 LNCaP cells were inoculated into the gland using a sterile syringe and 25 gauge needle. Such inoculation resulted a small swelling at the site. LNCaP cells under such condition give rise to tumors in 100% animals as observed in a pilot experiment. Abdominal incision was closed with sterile stainless steel clips. A group of 4 animals was returned to a cage following recovery from the sedation and recruited for the experiment. The treatment protocol was initiated 7 days after the intraprostatic inoculation of LNCaP cells, a time interval sufficient to give rise to small tumors as observed in the pilot experiment. A dose of 25 μg ARR_2PB-p202 plasmid DNA entrapped in a lipid formulation (SN) [27] at the ratio of 1:1.5 was incubated at room temperature for 30 min. The DNA/liposome complex was intravenously injected into tail vein. The mice were treated twice a week for a period of one and a half-month followed by once a week. The luciferase control group received equivalent dose of plasmid DNA (ARR_2PB-Luc)/liposome complex. Animals were examined weekly to assess the tumor growth.

Western Blot Analysis. Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM Na_2PO_4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na_3VO_4, 5 mM PMSF, 1% aprotinin, and 10 μg/ml leupeptin. The antibodies specific for human Rb, cyclin B, p55cdc, and actin (Santa Cruz Biotechnology, Inc.) were used to detect these proteins by western blot as described previously [20].

RESULTS AND DISCUSSION

p202 suppresses tumorigenicity of prostate cancer cells.

To investigate p202 could exhibit growth inhibitory effect on prostate tumor *in vivo*, two assays were performed. First, assay employed two p202 stable cell lines derived from human prostate cancer cell line, PC-3 [22]. Second, an *ex vivo* tumorigenicity assay using PC-3 cells transfected with p202. As shown in Fig. 1A, sixteen weeks post-implantation, p202-1 and p202-2 clones generated smaller tumors than that of the control, pcDNA3-pooled cell line. In fact, p202-2 clone failed to form tumors in mice under identical experimental set up. The difference in tumorigenesis between p202-1 and p202-2 may be attributed to an inadequate p202 protein expression in the former [22]. To rule out the possible contribution of clonal heterogeneity in the observed effects, we performed an *ex vivo* tumorigenicity assay in which PC-3 cells were transiently transfected with a p202 expression vector using two delivery vector systems via a polymer (PEI) and a liposome (LPD-1). The transfected PC-3 cells were employed to generate subcutaneous xenografts in nude mice. The p202 transfected PC-3 cells interestingly showed no detectable tumor after 10 days, regardless of the vector system used (Fig. 1B). On the contrary, the DNA

control, i.e., p202 alone, was ineffective in containing tumor growth, indicating that the observed anti-tumor effect on PC-3 cells is attributable to p202 transfection. The vector controls, i.e., PEI alone or LPD-1 alone, did not significantly affect tumor formation. Together, these results strongly suggest that p202 possess an anti-tumor activity against prostate cancer cells. Importantly, it provides a scientific basis for developing a p202-based gene therapy strategy in human prostate cancer xenograft model.

ARR_2PB promoter directs prostate-specific p202 expression and tumor suppression. To achieve prostate specific p202-mediated anti-tumor activity, we first tested whether an AR-responsive promoter could direct a luciferase (Luc) reporter gene expression in prostate. Since ARR_2PB promoter contains two copies of androgen response regions (ARR) located upstream from a minimum PB promoter, it is highly responsive to androgen-dependent transcriptional activation [23]. We generated ARR_2PB-luc and transfected it into two prostate cancer cell lines with (LNCaP) or without (PC-3) endogenous AR expression. We used a pancreatic cancer cell line, Panc-1, as a non-prostatic control cell. As shown in Fig. 2A, compared with the transfection of a luciferase gene expression vector driven by a constitutively active CMV promoter (CMV-Luc), the relative luciferase activity of ARR_2PB-luc/CMV-Luc is the highest in the AR-positive LNCaP cells, but not in AR-negative PC-3 and Panc-1 cells. This result suggests that ARR_2PB promoter activity is indeed AR-dependent [23], and thus confirms the utility of ARR_2PB promoter to direct AR-specific gene expression in prostate cancer cells [24, 25].

To test the AR-specific p202-mediated anti-tumor activity in prostate cancer xenograft model, we generated a p202 expression vector driven by ARR_2PB promoter (ARR_2PB-p202). Likewise, ARR_2PB-Luc served as a negative control. The prostate cancer xenograft model was established according to the procedure described previously [28, 29]. Initiation of treatment 7 days after tumor cell implantation in prostate prolonged survival in mice treated by ARR_2PB-p202. While all mice treated with ARR_2PB-luc were sacrificed on the 108th day post-treatment, as they carried massive tumors, exceeding the institutional permissible limit (Table 1), 100% of ARR_2PB-p202-treated mice were healthy and alive. Sixty percent (3 mice) of the ARR_2PB-p202-treated mice survived on the 150th day post-treatment. To assess the anti-tumor activity, in an interim sacrifice protocol, 3 mice each from ARR_2PB-p202 and ARR_2PB-luc treatment groups were euthanized and prostate glands dissected at day 77 of treatment. ARR_2PB-p202-treated tumors were remarkably reduced in size than those treated by the control vector, ARR_2PB-luc (Fig. 2B). This observation explains the prolonged survival seen in mice treated by ARR_2PB-p202. The use of ARR_2PB promoter to direct expression of p202 predicts the specificity of effect. Therefore, we examined the p202 expression on tumors and organs isolated from ARR_2PB-p202-treated mice by immunohistochemical staining. p202 expression was readily detected in tumors (Fig. 3A). The protein was detected in the cytoplasm as a red colored reaction product from the enzymatic reaction using aminoethylcarbazole as the chromogen. Note abundant intracytoplasmic expression of p202 in tumor from mouse treated with ARR_2PB-p202 (left panel). Mouse treated with ARR_2PB-Luc had undetectable p202 (right panel). Given that p202 is primarily a nuclear protein [30], the exact reason for the predominant cytoplasmic staining of p202 is not clear.

However, it is likely due to the robust expression of p202 that causes accumulation of p202 in the cytoplasm. Alternatively, since the induced p202 localizes in the cytoplasm for 30-36 h after IFN treatment before translocated into the nucleus [30], it is likely that p202 could still remain in the cytoplasm 20-h post ARR_2PB-p202 treatment. We also examined the organ-specific expression of p202 in multiple organs such as lung, liver, kidney, and heart. There was no extraprostatic expression of p202 except the reticuloendothelial cells of lung and liver (Fig. 3B). The p202-positive mouse reticuloendothelial cells is likely the endogenous level of p202 expression since all 200 amino-acid protein family members are expressed in hematopoietic cells [31]. Together, the results strongly suggest that systemic delivery of ARR_2PB-directed expression vector by SN liposome could result in prostate and AR-specific anti-tumor activity in prostate cancer.

p202 up-regulates the hypophosphorylated Rb and down-regulates cyclin B and p55cdc.

To investigate the underlying mechanisms of the p202-mediated growth inhibition and tumor suppression in prostate cancer cells, we set out to determine if (1) Rb phosphorylation was involved in p202-mediated growth arrest since IFN treatment increases the level of hypophosphorylated (active) form of Rb [32-34]; and (2) other regulatory genes responsible for the p202-mediated growth retardation and tumor suppression that can be identified by DNA array technology. To examine the effect on Rb phosphorylation by p202, we employed western blot using a Rb-specific antibody to analyze the phosphorylation status of Rb in both parental and p202-expressing prostate

cancer cells. Fig. 4A shows that the p202-expressing cells, i.e., p202-1, -2, and -3, exhibit an elevated level of hypophosphorylated form (faster migrating band) of Rb as compared to the control, i.e., pcDNA3-pooled, in which the hyperphosphorylated form (slower migrating band) of Rb is most prevalent. Thus, one possible mechanism by which p202 induces cell growth arrest in PC-3 is by enhancing the level of hypophosphorylated Rb. Presumably, the active Rb would then inhibit E2F transactivation function by forming a Rb/E2F complex. Thus, the E2F-mediated transcription of S-phase genes might be inhibited causing G1-phase arrest. Since p202 is a transcription modulator, it is possible that p202 could regulate certain gene expression which might be important in p202-mediated growth arrest and tumor suppression in prostate cancer cells. To identify other critical genes involved in p202-mediated anti-growth and anti-tumor activities, we employed DNA array technology. Using RNA products obtained from PC-3 (parental control) and p202-2 (a representative p202-expressing prostate cancer cell) to hybridize with DNA array filters (Clontech) containing 588 known genes that are involved in various cellular regulatory pathways including those of cell cycle control, we were able to identify several candidate genes whose expressions were found significantly influenced by p202 expression. Two such genes have been confirmed by western blot, i.e., cyclin B and p55cdc (Fig. 4B), which showed a reduced level of expression in p202-expressing cells, as compared to the control, pcDNA3-pooled. The sample loading was similar as indicated by the actin control. In light of the well documented p202-mediated G1 arrest, the reduction of cyclin B and p55cdc in p202-expressing cells is rather surprising since cyclin B is involved in G2/M phase transition [35] and p55cdc is required for normal metaphase-to-anaphase transition

involved in late mitotic events [36, 37]. It is likely that the down-regulation of these two genes by p202 may contribute to the p202-mediated cell cycle arrest. This is the first time that p202 is implicated in involvement in G2/M phase cell cycle control. It is possible that the p202-associated cyclin B and p55cdc down-regulation may contribute, in part, to the p202-mediated growth arrest.

In this report, we showed that p202 expression suppresses the tumorigenicity of prostate cancer cells. A subsequent *ex vivo* experiment using either CMV-p202/PEI or CMV-p202/LPD-1 complex also inhibited prostate cancer cell growth in xenograft model. The utility of p202 as a potential therapeutic gene for prostate cancer treatment was demonstrated by the observation that prostate-specific anti-tumor activity can be achieved by systemically treating the prostate tumor-bearing mice with a p202 expression vector driven by a composite probasin promoter, ARR_2PB. Thus, in addition to local and systemic treatment of breast and pancreatic tumors, respectively, by using a p202 expression vector driven by a constitutively active promoter such as CMV promoter [20, 21], our results suggest the feasibility of using a tissue-specific promoter to achieve p202-mediated anti-tumor activity in those cancer types as well. Experiments are underway to test that possibility. Given that p202 is involved in G0/G1 transition by targeting E2F/Rb pathway [17], it is interesting to note that G2/M cell cycle regulators such as cyclin B and p55cdc are down-regulated by p202. This observation suggests that p202 may also be involved in G2/M phase transition. This notion is supported by the apparent lack of accumulation of asynchronized cells in any specific phase of cell cycle of either p202 stable cells (data not shown) or p202 inducible cells [38]. Further characterization of p202

effect on G2/M transition and elucidation of the mechanism by which p202 down-regulates cyclin B and p55cdc will shed light onto how p202 uses the two-pronged pathway to inhibit cell proliferation.

APPENDIX-REFERENCES

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FIGURE LEGENDS

Fig. 1: The anti-tumor function of p202 in PC-3 cells. A) Reduced tumorigenicity of p202-expressing PC-3 cells. Nude mice were injected subcutaneously with 1 x 10^6 cells in each of the two sides on the abdomen. Tumor size was measured each week and the tumor volume was calculated. B) p202 reduces the tumorigenicity of PC-3 cells *ex vivo*. PC-cells were transfected with CMV-p202 using either PEI or LPD liposome, or without liposome (DNA control). Eighteen hours after transfection, 1 x 10^6 cells were s.c. injected in both sides on the abdomen of a nude mouse.

Fig. 2. A) ARR_2PB promoter activity is AR-dependent. ARR_2PB-luc (pb-luc) (0.5 mg) and CMV-luc (0.5 mg) were transfected into two prostate cancer cell lines with, e.g., LNCaP, or without, e.g., PC3, endogenous AR expression. A pancreatic cancer cell line, Panc-1, served as a non-prostatic cell control. The ratios of luciferase activity resulted from ARR_2PB-luc and CMV-luc transfections were measured. pRL-TK (50 ng) was co-transfected and served as an internal control for transfection efficiency using dual luciferase assay (Promega). The data shown here is the average of two independent experiments. B) Anti-tumor activity by systemic ARR_2PB-p202 treatment. Tumor suppression by ARR_2PB-p202 treatment. Representative LNCaP tumors (n = 3 per treatment group) are shown from mice treated with ARR_2PB-p202 or ARR_2PB-luc/SN liposome complexes on day 77 post treatment.

Fig. 3. ARR_2PB promoter directs prostate-specific p202 expression. A) Formalin fixed tumors from mice 20-h post-treatment were sectioned and stained for p202 employing

polyclonal anti-p202 antibodies as described under 'Materials and methods'. The protein was detected in the cytoplasm as a red colored reaction product from the enzymatic reaction using aminoethylcarbazole as the chromogen. Note abundant intracytoplasmic expression of p202 in tumor from mouse treated with ARR_2PB-p202 (left panel). Mouse treated with ARR_2PB-Luc had undetectable p202 (right panel). B) p202 expression was organ specific. Multiple organs from mice intravenously treated with ARR_2PB-p202 were examined for the expression of p202. There was no extraprostatic expression of p202 except the reticuloendothelial cells of lung and liver.

Fig. 4. p202 enhances hypophosphorylated Rb and reduces cyclin B and p55CDC expression. Cell lysates obtained from pcDNA3-pool and p202-expressing PC-3 cell lines (p202-1, -2, -3, and -4) were separated by SDS-PAGE and subsequently analyzed by western blot with antibodies against Rb, cyclin B, p55cdc, and actin. The actin bands serve as loading control.

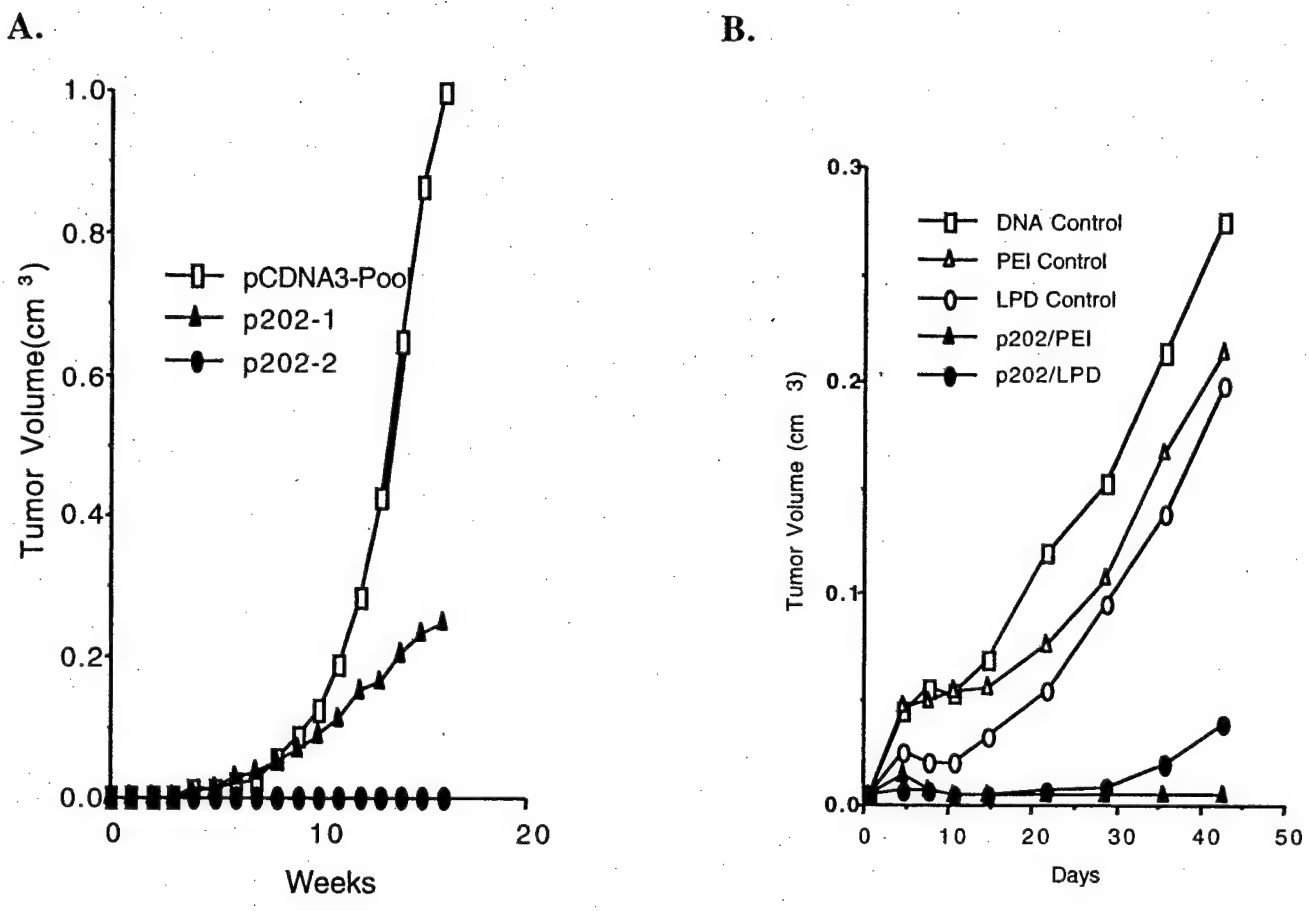


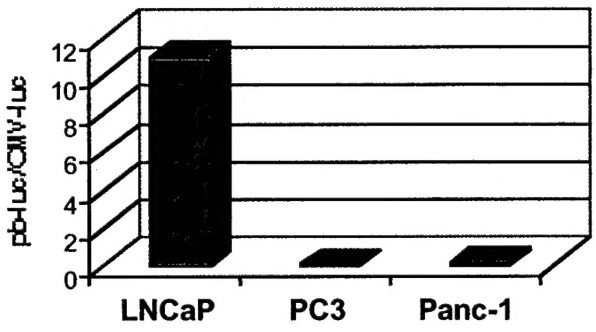
Figure 1: The anti-tumor function of p202 in PC-3 cells.
A. Reduced tumorignicity of p202-expressing PC-3 cells;
B. P202 reduces tumorigenicity of PC-3 cells *ex vivo*

Table-1: Survival data for mice bearing LNCaP cell tumors orthotopically and treated intravenously with either ARR_2PB-p202 or ARR_2PB-luc.

Group	# of mice*	Treatment	Survival(%) on day						
			80	100	110	120	130	140	150
I	8	ARR_2PB-luc	100	100	0	0	0	0	0
II	8	ARR_2PB-p202	100	100	100	100	100	80	60

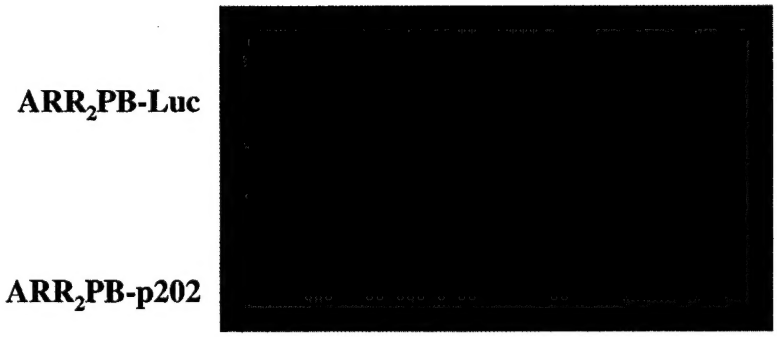
* Three mice from each group were sacrificed for tumor analysis on day 77

A.



ARR_2PB promoter activity is AR-dependent

B.

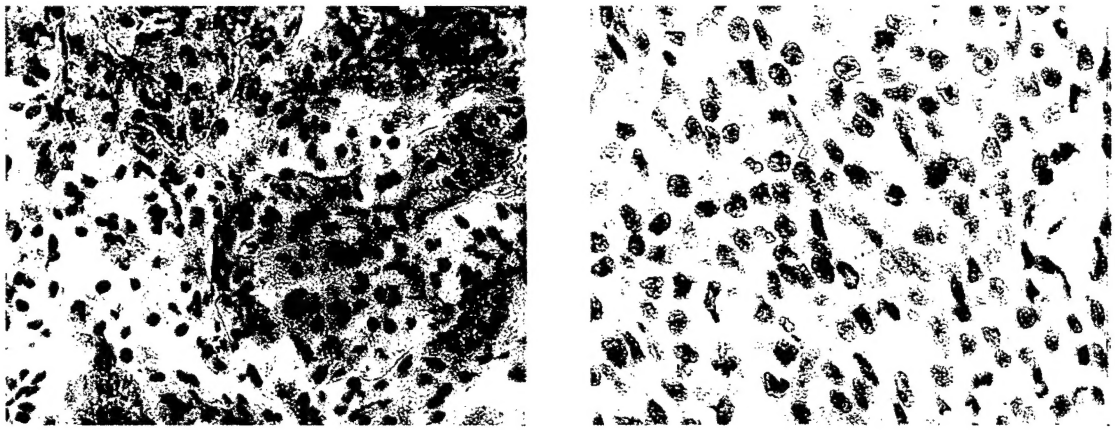


Anti-tumor activity by systemic ARR_2PB-p202 treatment

Figure 2

ARR2-PB-p202 **ARR2-PB-Luc**

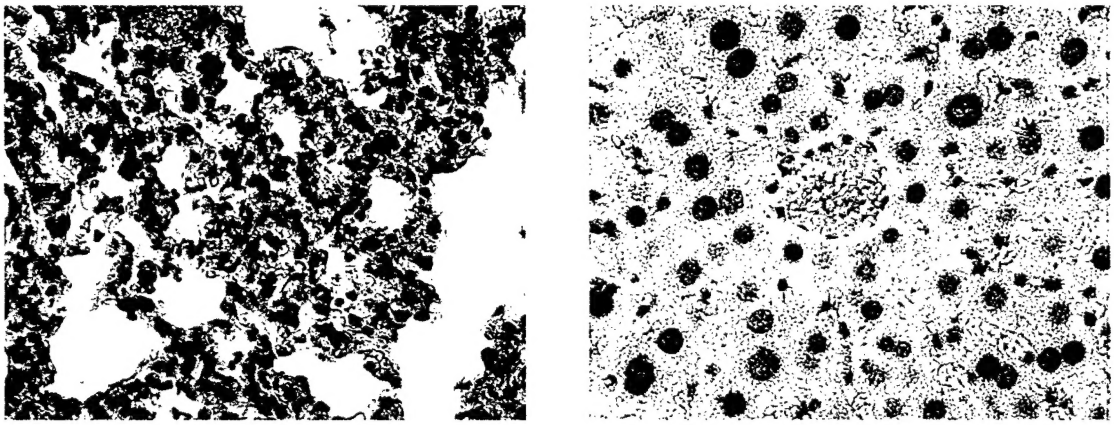
A



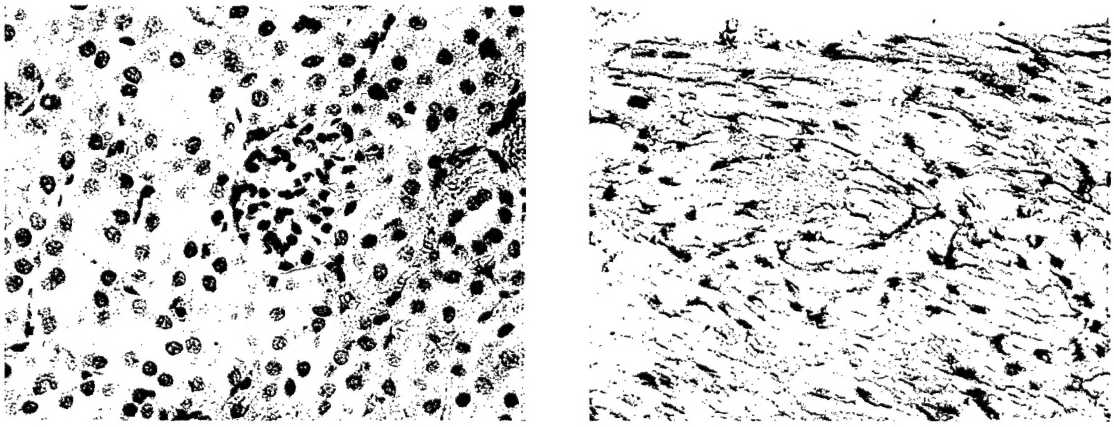
ARR2PB promoter directs prostate-specific p202 expression

B

Lung **Liver**



Kidney **Heart**



P202 expression is organ specific

Figure 3

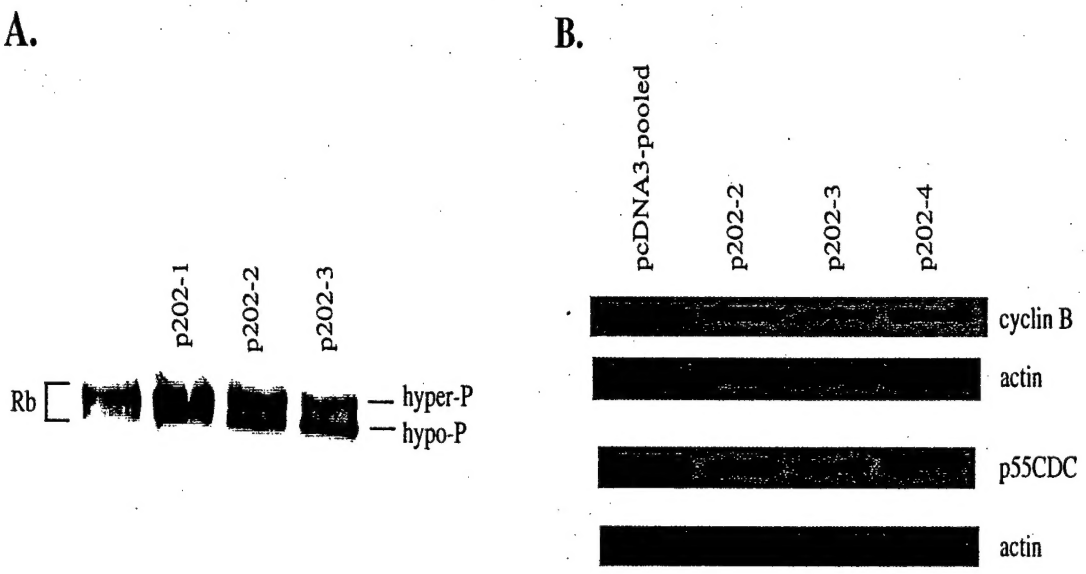


Figure 4: p202 enhances hypophosphorylated Rb and reduces cyclin B and p55 CDC expression